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(71) Applicant: QUEEN'S UNIVERSITY AT KINGSTON [CA/CA]; Kingston, Ontario K7L 3N6 (CA).

(72) Inventors: KISILEVSKY, Robert; 120 Cairn Grove, Kingston, Ontario K7M 4B9 (CA). SZAREK, Walter, A.; 403-322 Brock Street, Kingston, Ontario K7L 1S9 (CA). WEAVER, Donald; 27 Collegeview Crescent, Kingston, Ontario K7M 7J8 (CA).

(74) Agent: HICKS, Richard, J.; Parteq Innovations, Queen's University, Kingston, Ontario K7L 3N6 (CA).

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(54) Title: METHOD FOR TREATING AMYLOIDOSIS

(57) Abstract

Therapeutic compounds and methods for inhibiting amyloid deposition in a subject, whatever its clinical setting, are described. Amyloid deposition is inhibited by the administration to a subject of an effective amount of a therapeutic compound comprising an anionic group and a carrier molecule, or a pharmaceutically acceptable salt thereof, such that an interaction between an amyloidogenic protein and a basement membrane constituent is inhibited. Preferred anionic groups are sulfonates and sulfates. Preferred carrier molecules include carbohydrates, polymers, peptides, peptide derivatives, aliphatic groups, alicyclic groups, heterocyclic groups, aromatic groups and combinations thereof.

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METHOD FOR TREATING AMYLOIDOSIS

Background of Invention

Amyloidosis refers to a pathological condition characterized by the presence of amyloid. Amyloid is a generic term referring to a group of diverse but specific extracellular protein deposits which are seen in a number of different diseases. Though diverse in their occurence, all amyloid deposits have common morphologic properties, stain with specific dyes (e.g., Congo red), and have a characteristic red-green birefringent appearance in polarized light after staining. They also share common ultrastructural features and common x-ray diffraction and infrared spectra.

Amyloidosis can be classified clinically as primary, secondary, familial and/or isolated. Primary amyloid appears *de novo* without any preceding disorder. Secondary amyloid is that form which appears as a complication of a previosly existing disorder. Familial amyloid is a genetically inherited form found in particular geographic populations. Isolated forms of amyloid are those that tend to involve a single organ system. Different amyloids are also characterized by the type of protein present in the deposit. For example, neurodegenerative diseases such as scrapie, bovine spongiform encephalitis, Creutzfeldt-Jakob disease and the like are characterized by the appearance and accumulation of a protease-resistant form of a prion protein (referred to as AScr or PrP-27) in the central nervous system. Similarly, Alzheimer's disease, another neurodegenerative disorder, is characterized by congophilic angiopathy, neuritic plaques and neurofibrillary tangles, all of which have the characteristics of amyloids. In this case, the plaques and blood vessel amyloid is formed by the beta protein. Other systemic diseases such as adult-onset diabetes, complications of long-term hemodialysis and sequelae of long-standing inflammation or plasma cell dyscrasias are characterized by the accumulation of amyloids systemically. In each of these cases, a different amyloidogenic protein is involved in amyloid deposition.

Once these amyloids have formed there is no known therapy or treatment to dissolve the deposits *in situ*. With the exception of familial Mediterranean Fever, where colchicine is used prophylactically, no therapy exists for any form of amyloidosis. There is, therefore, an urgent need for therapeutic agents which can either inhibit the formation or growth of amyloid or dissolve amyloid deposits once formed.

Summary of the Invention

This invention pertains to methods and compositions useful for treating amyloidosis. The methods of the invention involve administering to a subject a therapeutic compound which inhibits amyloid deposition. Accordingly, the compositions and methods of the invention are useful for inhibiting amyloidosis in disorders in which amyloid deposition occurs. The methods of the invention can be used therapeutically to treat amyloidosis or can be used prophylactically in a subject susceptible to amyloidosis. The

methods of the invention are based, at least in part, on inhibiting an interaction between an amyloidogenic protein and a constituent of basement membrane to inhibit amyloid deposition. The constituent of basement membrane is a glycoprotein or proteoglycan, preferably heparan sulfate proteoglycan. A therapeutic compound used in the method of the invention can interfere with binding of a basement membrane constituent to a target binding site on an amyloidogenic protein, thereby inhibiting amyloid deposition.

In one embodiment, the method of the invention involves administering to a subject a therapeutic compound having at least one anionic group covalently attached to a carrier molecule which is capable of inhibiting an interaction between an amyloidogenic protein and a glycoprotein or proteoglycan constituent of a basement membrane to inhibit amyloid deposition. In one embodiment, the anionic group covalently attached to the carrier molecule is a sulfonate group. Accordingly, the therapeutic compound can have the formula :

$$Q—[—SO_3^-X^+]_n$$

wherein Q is a carrier molecule; X^+ is a cationic group; and n is an integer. In another embodiment, the anionic group is a sulfate group. Accordingly, the therapeutic compound can have the formula :

$$Q—[—OSO_3^-X^+]_n$$

wherein Q is a carrier molecule; X^+ is a cationic group; and n is an integer. Carrier molecules which can be used include carbohydrates, polymers, peptides, peptide derivatives, aliphatic groups, alicyclic groups, heterocyclic groups, aromatic groups and combinations thereof. Preferred therapeutic compounds for use in the invention include poly(vinyl sulfonic acid), ethanesulfonic acid, sucrose octasulfate, or pharmaceutically acceptable salts thereof.

The therapeutic compounds of the invention are administered to a subject by a route which is effective for inhibition of amyloid deposition. Suitable routes of administration include subcutaneous, intravenous and intraperitoneal injection. The therapeutic compounds of the invention have been found to be effective when administered orally. Accordingly, a preferred route of administration is oral administration. The therapeutic compounds can be administered with a pharmaceutically acceptable vehicle.

The invention further provides pharmaceutical compositions for treating amyloidosis. The pharmaceutical compositions include a therapeutic compound of the invention in an amount effective to inhibit amyloid deposition and a pharmaceutically acceptable vehicle.

Brief Description of Drawings

Figure 1 is a bar graph illustrating the effect of poly (vinyl sulfonic acid sodium salt) administered intraperitoneally on *in vivo* AA amyloid deposition in mouse spleen.

Figure 2 is a graph illustrating the effect of poly (vinyl sulfonic acid sodium salt) on heparan sulfate proteoglycan binding to β-APP in tris-buffered saline (TBS).

Figure 3 is a graph illustrating the effect of poly (vinyl sulfonic acid sodium salt) on heparan sulfate proteoglycan binding to β-APP in phosphate buffered saline (PBS).

Figure 4 is a bar graph illustrating the effect of poly(vinyl sulfonate) administered orally on *in vivo* AA amyloid deposition in mouse spleen.

Figure 5 is a graph illustrating the blood level of the amyloid precursor, SAA, over time for animals receiving poly(vinyl sulfonic acid sodium salt) (open circles) and control animals (triangles).

Figure 6 is a graph illustrating the effect of orally administered poly(vinyl sulfonic acid sodium salt) on the course of AA amyloid deposition in mouse spleen when amyloid deposits were already present prior to treatment of the animals. The triangles represent the control animals and the open circles represent the treated animals.

Figure 7 is a graph illustrating the effect of orally administered poly(vinyl sulfonic acid sodium salt) on splenic amyloid deposition when the inflammatory stimulus is maintained during the course of the experiment. The triangles represent the control animals and the open circles represent the treated animals.

Figure 8 is a graph illustrating the effect of orally administered ethane monosulfonate, sodium salt (EMS) on *in vivo* AA splenic amyloid deposition. The triangles represent the control animals, the open circles represent animals receiving 2.5 mg/ml of EMS in their drinking water, and the open squares represent animals receiving 6 mg/ml of EMS in their drinking water.

Detailed Description of Invention

This invention pertains to methods and compositions useful for treating amyloidosis. The methods of the invention involve administering to a subject a therapeutic compound which inhibits amyloid deposition. "Inhibition of amyloid deposition" is intended to emcompass prevention of amyloid formation, inhibition of further amyloid deposition in a subject with ongoing amyloidosis and reduction of amyloid deposits in a subject with ongoing amyloidosis. Inhibition of amyloid deposition is determined relative to an untreated subject or relative to the treated subject prior to treatment. Amyloid deposition is inhibited by inhibiting an interaction between an amyloidogenic protein and a constituent of basement membrane. "Basement membrane" refers to an extracellular matrix comprising glycoproteins and proteoglycans, including laminin, collagen type IV, fibronectin and heparan sulfate

proteoglycan (HSPG). In one embodiment, amyloid deposition is inhibited by interfering with an interaction between an amyloidogeic protein and a sulfated glycosaminoglycan such as HSPG. Sulfated glycosaminoglycans are known to be present in all types of amyloids (see Snow, A.D. et al. (1987) *Lab. Invest.* 56:120-123) and amyloid deposition and HSPG deposition occur coincidentally in animal models of amyloidosis (see Snow, A.D. et al. (1987) *Lab. Invest.* 56:665-675). In the methods of the invention, molecules which have a similar structure to a sulfated glycosaminoglycan are used to inhibit an interaction between an amyloidogenic protein and basement membrane constituent. In particular, the therapeutic compounds of the invention comprise at least one sulfate group or a functional equivalent thereof, for example a sulfonate group or other functionally equivalent anionic group, linked to a carrier molecule. In addition to functioning as a carrier for the anionic functionality, the carrier molecule can enable the compound to traverse biological membranes and to be biodistributed without excessive or premature metabolism. Moreover, when multiple anionic functionalities are present on a carrier molecule, the carrier molecule serves to space the anionic groups in a correct geometric separation.

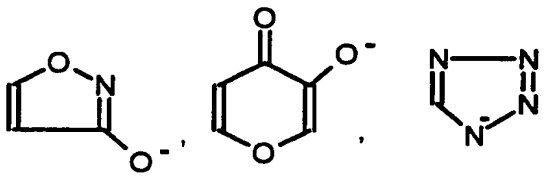
In one embodiment, the method of the invention includes administering to the subject an effective amount of a therapeutic compound which has at least one anionic group covalently attached to a carrier molecule. The therapeutic compound is capable of inhibiting an interaction between an amyloidogenic protein and a glycoprotein or proteoglycan constituent of a basement membrane to thus inhibit amyloid deposition. The therapeutic compound can have the formula :

$$Q—[—Y^-X^+]_n$$

wherein Y^- is an anionic group at physiological pH; Q is a carrier molecule; X^+ is a cationic group; and n is an integer. The number of anionic groups ("n") is selected such that the biodistribution of the compound for an intended target site is not prevented while maintaining activity of the compound. For example, the number of anionic groups is not so great as to inhibit traversal of an anatomical barrier, such as a cell membrane, or entry across a physiological barrier, such as the blood-brain barrier, in situations where such properties are desired. In one embodiment, n is an integer between 1 and 10. In another embodiment, n is an integer between 3 and 8.

An anionic group of a therapeutic compound of the invention is a negatively charged moieties that, when attached to a carrier molecule, can inhibit an interaction between an amyloidogenic protein and a glycoprotein or proteoglycan constituent of a basement membrane to thus inhibit amyloid deposition. For purposes of this invention, the anionic group is negatively charged at physiological pH. Preferably, the anionic therapeutic compound mimics the structure of a sulfated proteoglycan, i.e., is a sulfated compound or a

functional equivalent thereof. "Functional equivalents" of sulfates are intended to include bioisosteres. Bioisosteres encompass both classical bioisosteric equivalents and non-classical bioisosteric equivalents. Classical and non-classical bioisosteres of sulfate groups are known in the art (see e.g. Silverman, R.B. *The Organic Chemistry of Drug Design and Drug Action*, Academic Press, Inc.:San Diego, CA, 1992, pp.19-23). Accordingly, a therapeutic compound of the invention can comprise at least one anionic group including sulfonates, sulfates, phophonates, phosphates, carboxylates, and heterocyclic groups of the following formulas:



Depending on the carrier molecule, more than one anionic group can be attached thereto. When more than one anionic group is attached to a carrier molecule, the multiple anionic groups can be the same structural group (a.g., all sulfonates) or, alternatively, a combination of different anionic groups can be used (e.g., sulfonates and sulfates, etc.).

The ability of a therapeutic compound of the invention to inhibit an interaction between an amyloidogenic protein and a glycoprotein or proteoglycan constituent of a basement membrane can be assessed by an *in vitro* binding assay, such as that described in the Exemplification or in U.S. Patent No. 5,164,295 by Kisilevsky et al. Briefly, a solid support such as a polystyrene microtiter plate is coated with an amyloidogenic protein (e.g., serum amyloid A protein or β-amyloid precursor protein (β-APP)) and any residual hyrophobic surfaces are blocked. The coated solid support is incubated with various concentrations of a constituent of basement membrane, preferably HSPG, either in the presence or absence of a compound to be tested. The solid support is washed extensively to remove unbound material. The binding of the basement membrane constituent (e.g., HSPG) to the amyloidogenic protein (e.g., β-APP) is then measured using an antibody directed against the basement membrane constituent which is conjugated to a detectable substance (e.g., an enzyme, such as alkaline phosphatase) by detecting the detectable substance. A compound which inhibits an interaction between an amyloidogenic protein and a glycoprotein or proteoglycan constituent of a basement membrane will reduce the amount of substance detected (e.g., will inhibit the amount of enzyme activity detected).

Preferably, a therapeutic compound of the invention interacts with a binding site for a basement membrane glycoprotein or proteoglycan in an amyloidogenic protein and thereby inhibits the binding of the amyloidogenic protein to the basement membrane consitituent. Basement membrane glycoproteins and proteoglycans include laminin, collagen type IV, fibronectin and heparan sulfate proteoglycan (HSPG). In a preferred embodiment,

the therapeutic compound inhibits an interaction between an amyloidogenic protein and HSPG. Consensus binding site motifs for HSPG in amyloidogenic proteins have been described (see e.g. Cardin and Weintraub (1989) *Arteriosclerosis* 9:21-32). For example, an HSPG consensus binding motif can be of the general formula X1-X2-Y-X3, wherein X1, X2 and X3 are basic amino acids (e.g., lysine or arginine) and Y is any amino acid. Modeling of the geometry of this site led to determination of the following spacing between basic amino acid residues (carboxylate to carboxylate, in Angstroms):

X1-X2	5.3 ± 1.5 Å
X1-X3	7.1 ± 1.5 Å
X2-X3	7.6 ± 1.5 Å

These values were determined using a combination of molecular mechanics and semi-empirical quantum mechanics calculations. Molecular mechanics calculations were performed using the MM2 force field equation. Semi-empirical molecular orbital calculations were performed using the AM1 Hamiltonian equation. The conformational space of the site was sampled using a combination of molecular dynamics (both high and low temperature) and Monte Carlo simulations.

Accordingly, in the therapeutic compounds of the invention, when multiple anionic groups are attached to a carrier molecule, the relative spacing of the anionic groups can be chosen such that the anionic groups (e.g., sulfonates) optimally interact with the basic residues within the HSPG binding site (thereby inhibiting interaction of HSPG with the site). For example, anionic groups can be spaced approximately 5.3 ± 1.5 Å, 7.1 ± 1.5 Å and/or 7.6 ± 1.5 Å apart, or appropriate multiples thereof, such that the relative spacing of the anionic groups allows for optimal interaction with a binding site for a basement membrane constituent (e.g., HSPG) in an amyloidogenic protein.

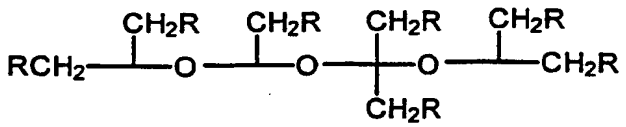
A therapeutic compound of the invention typically further comprises a counter cation (i.e., X^+ in the general formula: $Q—[—Y^-X^+]_n$). Cationic groups include positively charged atoms and moieties. If the cationic group is hydrogen, H^+, then the compound is considered an acid, e.g., ethanesulfonic acid. If hydrogen is replaced by a metal or its equivalent, the compound is a salt of the acid. Pharmaceutically acceptable salts of the therapeutic compound are within the scope of the invention. For example, X^+ can be a pharmaceutically acceptable alkali metal, alkaline earth, higher valency cation (e.g., aluminum salt), polycationic counter ion or ammonium. A preferred pharmaceutically acceptable salt is a sodium salt but other salts are also contemplated within their pharmaceutically acceptable range.

Within the therapeutic compound, the anionic group(s) is covalently attached to a carrier molecule. Suitable carrier molecules include carbohydrates, polymers, peptides,

peptide derivatives, aliphatic groups, alicyclic groups, heterocyclic groups, aromatic groups or combinations thereof. A carrier molecule can be substituted, e.g. with one or more amino, nitro, halogen, thiol or hydroxy groups.

As used herein, the term "carbohydrate" is intended to include substituted and unsubstituted mono-, oligo-, and polysaccharides. Monosaccharides are simple sugars usually of the formula $C_6H_{12}O_6$ that can be combined to form oligosaccharides or polysaccharides. Monosaccharides include enantiomers and both the D and L stereoisomers of monosaccharides. Carbohydrates can have multiple anionic groups attached to each monosaccharide moiety. For example, in sucrose octasulfate, four sulfate groups are attached to each of the two monosaccharide moieties.

As used herein, the term "polymer" is intended to include molecules formed by the chemical union of two or more combining subunits called monomers. Monomers are molecules or compounds which usually contain carbon and are of relatively low molecular weight and simple structure. A monomer can be converted to a polymer by combination with itself or other similar molecules or compounds. A polymer may be composed of a single identical repeating subunit or multiple different repeating subunits (copolymers). Polymers within the scope of this invention include substituted and unsubstituted vinyl, acryl, styrene and carbohydrate-derived polymers and copolymers and salts thereof. In one embodiment, the polymer has a molecular weight of approximately 800-1000 Daltons. Examples of polymers with suitable covalently attached anionic groups (e.g., sulfonates or sulfates) include poly(2-acrylamido-2-methyl-1-propanesulfonic acid); poly(2-acrylamido-2-methyl-1-propanesulfonic acid-co-acrylonitrile); poly(2-acrylamido-2-methyl-1-propanesulfonic acid-co-styrene); poly(vinylsulfuric acid); poly(sodium 4-styrenesulfonic acid); and sulfates and sulfonates derived from: poly(acrylic acid); poly(methyl acrylate); poly(methyl methacrylate); and poly(vinyl alcohol); and pharmaceutically acceptable salts thereof. Examples of carbohydrate-derived polymers with suitable covalently attached anionic groups include those of the formula:



wherein R is SO_3^- or OSO_3^-; and pharmaceutically acceptable salts thereof.

Peptides and peptide derivatives can also act as carrier molecules. The term "peptide" includes two or more amino acids covalently attached through a peptide bond. Amino acids which can be used in peptide carrier molecules include those naturally occurring amino acids found in proteins such as glycine, alanine, valine, cysteine, leucine, isoleucine, serine, threonine, methionine, glutamic acid, aspartic acid, glutamine, asparagine, lysine,

arginine, proline, histidine, phenylalanine, tyrosine, and tryptophan. The term amino acid further includes analogs, derivatives and congeners of naturally occurring amino acids, one or more of which can be present in a peptide derivative. For example, amino acid analogs can have a lengthened or shortened side chains or variant side chains with appropriate functional groups. Also included are the D and L stereoisomers of an amino acid when the structure of the amino acid admits of stereoisomeric forms. The term "peptide derivative" further includes compounds which contain molecules which mimic a peptide backbone but are not amino acids (so-called peptidomimetics), such as benzodiazepine molecules (see e.g. James, G. L. et al. (1993) *Science* 260:1937-1942). The anionic groups can be attached to a peptide or peptide derivative through a functional group on the side chain of certain amino acids or other suitable functional group. For example, a sulfate or sulfonate group can be be attached through the hydroxy side chain of a serine residue. A peptide can be designed to interact with a binding site for a basement membrane constituent (e.g., HSPG) in an amyloidogenic protein (as described above). Accordingly, in one embodiment, the peptide is comprised of four amino acids and anionic groups (e.g., sulfonates) are attached to the first, second and fourth amino acid. For example, the peptide can be Ser-Ser-Y-Ser, wherein an anionic group is attached to the side chain of each serine residue and Y is any amino acid. In addition to peptides and peptide derivatives, single amino acids can be used as carriers in the therapeutic compounds of the invention. For example, cysteic acid, the sulfonic acid derivative of cysteine, can be used.

The term "aliphatic group" is intended to include organic compounds characterized by straight or branched chains, typically having between 1 and 22 carbon atoms. Aliphatic groups include alkyl groups, alkenyl groups and alkynyl groups. In complex structures, the chains can be branched or cross-linked. Alkyl groups include saturated hydrocarbons having one or more carbon atoms, including straight-chain alkyl groups and branched-chain alkyl groups. Such hydrocarbon moieties may be substituted on one or more carbons with, for example, a halogen, a hydroxyl, a thiol, an amino, an alkoxy, an alkylcarboxy, an alkylthio, or a nitro group. Unless the number of carbons is otherwise specified, "lower aliphatic" as used herein means an aliphatic group, as defined above (e.g., lower alkyl, lower alkenyl, lower alkynyl), but having from one to six carbon atoms. Representative of such lower aliphatic groups, e.g., lower alkyl groups, are methyl, ethyl, n-propyl, isopropyl, 2-chloropropyl, n-butyl, sec-butyl, 2-aminobutyl, isobutyl, tert-butyl, 3-thiopentyl, and the like. As used herein, the term "amino" means $-NH_2$; the term "nitro" means $-NO_2$; the term "halogen" designates -F, -Cl, -Br or -I; the term "thiol" means SH_2; and the term "hydroxyl" means -OH. Thus, the term "alkylamino" as used herein means an alkyl group, as defined above, having an amino group attached thereto. The term "alkylthio" refers to an alkyl group, as defined above, having a sulphydryl group attached thereto. The term "alkylcarboxyl" as used herein means an alkyl group, as defined above, having a

carboxyl group attached thereto. The term "alkoxy" as used herein means an alkyl group, as defined above, having an oxygen atom, attached thereto. Representative alkoxy groups include methoxy, ethoxy, propoxy, tert-butoxy and the like.

The terms "alkenyl" and "alkynyl" refer to unsaturated aliphatic groups analogous to alkyls, but which contain at least one double or triple bond respectively.

The term "alicyclic group" is intended to include closed ring structures of three or more carbon atoms. Alicyclic groups include cylcoparaffins or naphthenes which are saturated cyclic hydrocarbons, cylcoolefins which are unsaturated with two or more double bonds, and cycloacetylenes which have a triple bond. They do not include aromatic groups. Examples of cycloparaffins include cyclopropane, cyclohexane, and cyclopentane. Examples of cycloolefins include cyclopentadiene and cyclooctatetrene. Alicyclic groups also include fused ring structures and substituted alicyclic groups such as alkyl substituted alicyclic groups. In the instance of the alicyclics such substituents can further comprise a lower alkyl, a lower alkenyl, a lower alkoxy, a lower alkylthio, a lower alkylamino, a lower alkylcarboxyl, a nitro, a hydroxyl, $-CF_3$, -CN, or the like.

The term "heterocyclic group" is intended to include closed ring structures in which one or more of the atoms in the ring is an element other than carbon, for example, nitrogen, or oxygen. Heterocyclic groups can be saturated or unsaturated and heterocyclic group such as pyrrole and furan can have aromatic character. They include fused ring structures such as quinoline and isoquinoline. Other examples of heterocyclic groups include pyridine and purine. Heterocyclic groups can also be substituted at one or more constituent atoms with, for example, a halogen, a lower alkyl, a lower alkenyl, a lower alkoxy, a lower alkylthio, a lower alkylamino, a lower alkylcarboxyl, a nitro, a hydroxyl, $-CF_3$, -CN, or the like.

The term "aromatic group" is intended to include unsaturated cyclic hydrocarbons containing one or more rings. Aromatic groups include 5- and 6-membered single-ring groups which may include from zero to four heteroatoms, for example, benzene, pyrrole, furan, thiophene, imidazole, oxazole, thiazole, triazole, pyrazole, pyridine, pyrazine, pyridazine and pyrimidine, and the like. The aromatic ring may be substituted at one or more ring positions with, for example, a halogen, a lower alkyl, a lower alkenyl, a lower alkoxy, a lower alkylthio, a lower alkylamino, a lower alkylcarboxyl, a nitro, a hydroxyl, $-CF_3$, -CN, or the like.

The therapeutic compound of the invention can be administered in a pharmaceutically acceptable vehicle. As used herein "pharmaceutically acceptable vehicle" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like which are compatible with the activity of the compound and are physiologically acceptable to the subject. An example of a pharmaceutically acceptable vehicle is buffered normal saline (0.15 molar NaCl). The use of

such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the therapeutic compound, use thereof in the compositions suitable for pharmaceutical administration is contemplated. Supplementary active compounds can also be incorporated into the compositions.

In a preferred embodiment of the method of the invention, the therapeutic compound administered to the subject is comprised of at least one sulfonate group covalently attached to a carrier molecule, or a pharmaceutically acceptable salt thereof. Accordingly, the therapeutic compound can have the formula:

$$Q—[—SO_3^-X^+]_n$$

wherein Q is a carrier molecule; X^+ is a cationic group; and n is an integer. Suitable carrier molecules and cationic groups are those described hereinbefore. The number of sulfonate groups ("n") is selected such that the biodistribution of the compound for an intended target site is not prevented while maintaining activity of the compound as discussed earlier. In one embodiment, n is an integer between 1 and 10. In another embodiment, n is an integer between 3 and 8. As described earlier, therapeutic compounds with multiple sulfonate groups can have the sulfonate groups spaced such that the compound interacts optimally with an HSPG binding site within an amyloidogenic protein.

In preferred embodiments, the carrier molecule for a sulfonate(s) is a lower aliphatic group (e.g., a lower alkyl, lower alkenyl or lower alkynyl), a disaccharide, a polymer or a peptide or peptide derivative. Furthermore, the carrier can be substituted, e.g. with one or more amino, nitro, halogen, thiol or hydroxy groups.

Examples of suitable sulfonated polymeric therapeutic compounds include poly(2-acrylamido-2-methyl-1-propanesulfonic acid); poly(2-acrylamido-2-methyl-1-propanesulfonic acid-co-acrylonitrile); poly(2-acrylamido-2-methyl-1-propanesulfonic acid-co-styrene); poly(vinylsulfonic acid); poly(sodium 4-styrenesulfonic acid); a sulfonate derivative of poly(acrylic acid); a sulfonate derivative of poly(methyl acrylate); a sulfonate derivative of poly(methyl methacrylate); and a sulfonate derivative of poly(vinyl alcohol); and pharmaceutically acceptable salts thereof.

A preferred sulfonated polymer is poly(vinyl sulfonic acid) (PVS) or a pharmaceutically acceptable salt thereof, preferably the sodium salt thereof. In one embodiment, PVS having a molecular weight of about 800-1000 Daltons is used . A preferred sulfonated disaccharide is a fully or partially sulfonated sucrose, or pharmaceutically acceptable salt thereof. Preferred low molecular weight (i.e., lower aliphatic) sulfonated compounds for use in the invention include ethanesulfonic acid; 2-amino-ethanesulfonic acid (taurine); cysteic acid (3-sulfoalanine or α-amino-β-

sulfopropionic acid); propane-1-sulfonic acid; ethane-1,2-disulfonic acid; propane-1,3-disulfonic acid, and butane-1,4-disulfonic acid; and pharmaceutically acceptable salts thereof.

In another embodiment of the method of the invention, the therapeutic compound administered to the subject is comprised of at least one sulfate group covalently attached to a carrier molecule, or a pharmaceutically acceptable salt thereof. Accordingly, the therapeutic compound can have the formula:

$$Q\text{—}[\text{—}OSO_3^-X^+]_n$$

wherein Q is a carrier molecule; X^+ is a cationic group; and n is an integer. Suitable carrier molecules and cationic groups are those described hereinbefore. The number of sulfate groups ("n") is selected such that the biodistribution of the compound for an intended target site is not prevented while maintaining activity of the compound as discussed earlier. In one embodiment, n is an integer between 1 and 10. In another embodiment, n is an integer between 3 and 8. As described earlier, therapeutic compounds with multiple sulfate groups can have the sulfate groups spaced such that the compound interacts optimally with an HSPG binding site within an amyloidogenic protein.

In preferred embodiments, the carrier molecule for a sulfate(s) is a lower aliphatic group (e.g., a lower alkyl, lower alkenyl or lower alkynyl), a disaccharide, a polymer or a peptide or peptide derivative. Furthermore, the carrier can be substituted, e.g. with one or more amino, nitro, halogen, thiol or hydroxy groups.

Examples of suitable sulfated polymeric therapeutic compounds include poly(2-acrylamido-2-methyl-1-propanesulfuric acid); poly(2-acrylamido-2-methyl-1-propanesulfuric acid-co-acrylonitrile); poly(2-acrylamido-2-methyl-1-propanesulfuric acid-co-styrene); poly(vinylsulfuric acid); poly(sodium 4-styrenesulfuric acid); a sulfate derivative of poly(acrylic acid); a sulfate derivative of poly(methyl acrylate); a sulfate derivative of poly(methyl methacrylate); and a sulfate derivative of poly(vinyl alcohol); and pharmaceutically acceptable salts thereof.

A preferred sulfated polymer is poly(vinyl sulfuric acid) or pharmaceutically acceptable salt thereof. A preferred sulfated disaccharide is sucrose octasulfate or pharmaceutically acceptable salt thereof. Preferred low molecular weight (i.e., lower aliphatic) sulfated compounds for use in the invention include ethanesulfuric acid; 2-aminoethanesulfuric acid; propane-1-sulfuric acid; ethane-1,2-disulfuric acid; propane-1,3-disulfuric acid and butane-1,4-disulfuric acid; and pharmaceutically acceptable salts thereof.

Dermatan sulfate and chondroitin 6-sulfate have not been observed to be effective in the method of the invention and therefore are not preferred for use in the invention.

A further aspect of the invention includes pharmaceutical compositions for treating amyloidosis. The therapeutic compounds in the methods of the invention, as described hereinbefore, can be incorporated into a pharmaceutical composition in an amount effective to inhibit amyloidosis in a pharmaceutically acceptable vehicle.

In one embodiment, the pharmaceutical compositions of the invention include a therapeutic compound that has at least one sulfonate group covalently attached to a carrier molecule, or a pharmaceutically acceptable salt thereof, in an amount sufficient to inhibit amyloid deposition, and a pharmaceutically acceptable vehicle. The therapeutic composition can have the formula:

$$Q\text{—}[\text{—}SO_3^-X^+]_n$$

wherein Q is a carrier molecule; X^+ is a cationic group; and n is an integer selected such that the biodistribution of the compound for an intended target site is not prevented while maintaining activity of the compound.

In another embodiment, the pharmaceutical compositions of the invention include a therapeutic compound that has at least one sulfate group covalently attached to a carrier molecule, or a pharmaceutically acceptable salt thereof, in an amount sufficient to inhibit amyloid deposition, and a pharmaceutically acceptable vehicle. The therapeutic compound can have the following formula:

$$Q\text{—}[\text{—}OSO_3^-X^+]_n$$

wherein Q is a carrier molecule; X^+ is a cationic group; and n is an integer selected such that the biodistribution of the compound for an intended target site is not prevented while maintaining activity of the compound.

Carrier molecule useful in the therapeutic compounds include carrier molecules previously described, e.g. carbohydrates, polymers, peptides, peptide derivatives, aliphatic groups, alicyclic groups, heterocyclic groups, aromatic groups or combinations thereof. Suitable polymers include substituted and unsubstituted vinyl, acryl, styrene and carbohydrate-derived polymers and copolymers and salts thereof. Preferred carrier molecules include a lower alkyl group, a dissacharide, a polymer or a peptide or peptide derivative.

In one embodiment, the therapeutic compound in the pharmaceutical compositions is a sulfonated polymer, for example poly(2-acrylamido-2-methyl-1-propanesulfonic acid); poly(2-acrylamido-2-methyl-1-propanesulfonic acid-co-acrylonitrile); poly(2-acrylamido-2-methyl-1-propanesulfonic acid-co-styrene); poly(vinylsulfonic acid); poly(sodium 4-styrenesulfonic acid); a sulfonate derivative of poly(acrylic acid); a sulfonate

derivative of poly(methyl acrylate); a sulfonate derivative of poly(methyl methacrylate); and a sulfonate derivative of poly(vinyl alcohol); and pharmaceutically acceptable salts thereof.

In another embodiment, the therapeutic compound in the pharmaceutical compositions is a sulfated polymer, for example poly(2-acrylamido-2-methyl-1-propanesulfuric acid); poly(2-acrylamido-2-methyl-1-propanesulfuric acid-co-acrylonitrile); poly(2-acrylamido-2-methyl-1-propanesulfuric acid-co-styrene); poly(vinylsulfuric acid); poly(sodium 4-styrenesulfuric acid); a sulfate derivative of poly(acrylic acid); a sulfate derivative of poly(methyl acrylate); a sulfate derivative of poly(methyl methacrylate); and a sulfate derivative of poly(vinyl alcohol); and pharmaceutically acceptable salts thereof.

Preferred therapeutic compounds for inclusion in a pharmaceutical composition for treating amyloidosis of the invention include poly(vinyl sulfuric acid); poly(vinyl sulfonic acid); sucrose octasulfate; a partially or fully sulfonated sucrose; ethanesulfuric acid; ethanesulfonic acid; 2-aminoethanesulfonic acid (taurine); 2-aminoethanesulfuric acid; cysteic acid (3-sulfoalanine or α-amino-β-sulfopropionic acid); propane-1-sulfonic acid; propane-1-sulfuric acid; ethane-1,2-disulfonic acid; ethane-1,2-disulfuric acid; propane-1,2-disulfonic acid; propane-1,2-disulfuric acid; butane-1,4-disulfonic acid; butane-1,4-disulfuric acid; and pharmaceutically acceptable salts thereof.

In the methods of the invention, amyloid deposition in a subject is inhibited by administering a therapeutic compound of the invention to the subject. The term subject is intended to include living organisms in which amyloidosis can occur. Examples of subjects include humans, monkeys, cows, sheep, goats, dogs, cats, mice, rats, and transgenic species thereof. Administration of the compositions of the present invention to a subject to be treated can be carried out using known procedures, at dosages and for periods of time effective to inhibit amyloid deposition in the subject. An effective amount of the therapeutic compound necessary to achieve a therapeutic effect may vary according to factors such as the amount of amyloid already deposited at the clinical site in the subject, the age, sex, and weight of the subject, and the ability of the therapeutic compound to inhibit amyloid deposition in the subject. Dosage regimens can be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation. A non-limiting example of an effective dose range for a therapeutic compound of the invention (e.g., poly(vinyl sulfonate sodium salt)) is between 5 and 500 mg/kg of body weight/per day.

As demonstrated in the Exemplification, the therapeutic compounds of the invention are effective when administered orally. Accordingly, a preferred route of administration is oral administration. Alternatively, the active compound (i.e., the therapeutic compound that can inhibit amyloid deposition) may be administered by other suitable routes such subcutaneous, intravenous, intraperitoneal, etc. administration (e.g. by injection). Depending on the route of administration, the active compound may be coated in

a material to protect the compound from the action of acids and other natural conditions which may inactivate the compound.

To administer the therapeutic compound by other than parenteral administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation. For example, the therapeutic compound may be administered to a subject in an appropriate carrier, for example, liposomes, or a diluent. Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Liposomes include water-in-oil-in-water CGF emulsions as well as conventional liposomes (Strejan *et al.*, (1984) *J. Neuroimmunol.* 7:27).

The therapeutic compound may also be administered parenterally or intraperitoneally. Dispersions can be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases, the composition must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The vehicle can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, sodium chloride, or polyalcohols such as mannitol and sorbitol, in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate or gelatin.

Sterile injectable solutions can be prepared by incorporating the therapeutic compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the therapeutic compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which

yields a powder of the active ingredient (i.e., the therapeutic compound) plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The therapeutic compound can be orally administered, for example, with an inert diluent or an assimilable edible carrier. The therapeutic compound and other ingredients may also be enclosed in a hard or soft shell gelatin capsule, compressed into tablets, or incorporated directly into the subject's diet. For oral therapeutic administration, the therapeutic compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. The percentage of the therapeutic compound in the compositions and preparations may, of course, be varied. The amount of the therapeutic compound in such therapeutically useful compositions is such that a suitable dosage will be obtained.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subjects to be treated; each unit containing a predetermined quantity of therapeutic compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical vehicle. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the therapeutic compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an therapeutic compound for the treatment of amyloid deposition in subjects.

The method of the invention is useful for treating amyloidosis associated with any disease in which amyloid deposition occurs. Clinically, amyloidosis can be primary, secondary, familial or isolated. Amyloids have been categorized by the type of amyloidogenic protein contained within the amyloid. Non-limiting examples of amyloids which can be inhibited, as identified by their amyloidogenic protein, are as follows (with the associated disease in parentheses after the amyloidogenic protein): β-amyloid (Alzheimer's disease, Down's syndrome, hereditary cerebral hemorrage amyloidosis [Dutch]); amyloid A (reactive [secondary] amyloidosis, familial Mediterranean Fever, familial amyloid nephrophathy with urticaria and deafness [Muckle-Wells syndrome]); amyloid κ L-chain or amyloid λ L-chain (idiopathic [primary], myeloma or macroglobulinemia-associated); Aβ2M (chronic hemodialysis); ATTR (familial amyloid polyneuropathy [Portuguese, Japanese, Swedish], familial amyloid cardiomyopathy [Danish], isolated cardiac amyloid, systemic senile amyloidosis); AIAPP or amylin (adult onset diabetes, insulinoma); atrial naturetic factor (isolated atrial amyloid); procalcitonin (medullary carcinoma of the thyroid); gelsolin (familial amyloidosis [Finnish]); cystatin C (hereditary cerebral hemorrage with amyloidosis [Icelandic]); AApoA-I (familial amyloidotic polyneuropathy [Iowa]); AApoA-II (accelerated senescence in mice); fibrinogen-associated amyloid; lysozyme-associated amyloid; and AScr

or PrP-27 (Scrapie, Creutzfeldt-Jacob disease, Gerstmann-Straussler-Scheinker syndrome, bovine spongiform encephalitis).

In certain embodiments of the invention, Congo red is excluded from sulfonated compounds used in the method of the invention.

In certain embodiments of the invention, the following sulfated compounds are excluded from use in the method of the invention: dextran sulfate 500, ι-carrageenan, λ-carrageenan, dextran sulfate 8, κ-carrageenan, pentosan polysulfate, and/or heparan.

In certain embodiments of the invention, the compositions and methods of the invention are used to inhibit amyloid deposition in amyloidosis wherein the amyloidogenic protein is not the protease-resistant form of a prion protein, AScr (also known as PrP-27).

The invention is further illustrated by the following examples which should not be construed as further limiting the subject invention. The contents of all references and published patent applications cited throughout this application are hereby incorporated by reference.

Exemplification

In the following experiments, a well-characterized mouse model of amyloidosis was used. In this *in vivo* system, animals receive an inflammatory stimulus and amyloid enhancing factor. For acute amyloidosis (i.e., short term amyloid deposition), the inflammatory stimulus is $AgNO_3$. For chronic amyloidosis (ongoing amyloid deposition), the inflammatory stimulus is lipopolysaccharide (LPS). Amyloid deposition (AA amyloid) in the spleens of mice was measured with and without therapeutic treatment.

Experiment 1

Animals

All mice were of the CD strain (Charles Rivers, Montreal, Quebec) and weighing 25-30 g.

Animal Treatment

All animals received $AgNO_3$ (0.5 ml, 2% solution) subcutaneously in the back, and amyloid enhancing factor (AEF) 100 μg intravenously. The preparation of amyloid enhancing factor has been described previously in Axelrad, M.A. *et al.* ("Further Characterization of Amyloid Enhancing Factor" *Lab. Invest.* 47:139-146 (1982)). The animals were divided into several groups one of which was an untreated control group which was sacrificed six days later. The remaining animals were divided into those which received

poly(vinyl sulfonic acid sodium salt) (PVS) at 50 mg, 40 mg, 20 mg, or 10mg by intraperitoneal injection every 12 hours or sucrose octasulfate ammonium salt (SOA) at 73 mg or 36.5 mg every 8 hours by IP injection. Surviving animals were sacrificed on the 5th day of treatment. In all cases the PVS or SOA was dissolved in a sterile aqueous carrier.

Tissue Preparation

At the termination of the experiments, the animals were sacrificed by cervical dislocation and the spleens, livers, and kidneys were fixed in 96% ethanol, 1% glacial acetic acid and 3% water as described in Lyon, A.W. *et al.* ("Co-deposition of Basement Membrane Components During the Induction of Murine Splenic AA Amyloid" *Lab. Invest.* 64:785-790 (1991)). Following fixation, the tissues were embedded in paraffin, 8-10 micron sections were cut and stained with Congo Red without counterstain as described in Puchtler, H. *et al.* ("Application of Thiazole Dyes to Amyloid Under Conditions of Direct Cotton Dyeing: Correlation of Histochemical and Chemical Data" *Histochemistry* 77:431-445 (1983)). The histologic sections viewed under polarized light were assessed by image analysis for the percent of spleen occupied by amyloid. In the case of the experiments with sucrose octasulfate, the tissues were immunostained with an antibody to the SAA protein (described in Lyon A.W. et al. *Lab Invest.* 64:785-790 (1991)) and the immunostained sections assessed by image analysis for the percent of tissue section occupied by amyloid.

Viability of Animals

All control animals survived the experiment without incident. In the case of the animals undergoing therapy, all animals given sucrose octasulfate at 73 mg/injection succumbed prior to the termination of the experiment. Animals receiving 36.5 mg of sucrose octasulfate/injection all survived. Of those animals receiving PVS (molecular weight 900-1000) in each dosage group, approximately half to one-third of the animals succumbed prior to the termination of the experiment. In all cases of animal deaths prior to the end of the experiments, the cause of death was uncontrolled intraperitoneal hemorrhage.

Effects of Agents on Amyloid Deposition

The effect of sucrose octasulfate at 36.5 mg/injection is shown below in Table 1. The mean area of spleen occupied by amyloid in control animals was 7.8% ± 1.5% S.E.M. In animals receiving the therapeutic agent the mean area was 3.2% ± 0.5% S.E.M. The difference is significant at a $p \leq 0.02$.

Table 1

Effect of Sucrose Octasulfate Ammonium Salt
on AA Amyloid Deposition *In vivo* in Mouse Spleen
% Area Occupied by Amyloid

Untreated	7.8 + 1.5	n = 5
Sucrose OctaSO_4	3.2 + 0.5	n = 5
	$p \leq 0.02$	

In the case of PVS, the data are shown in Figure 1. There was a profound inhibition of amyloid deposition at all doses with the suggestion of a dose-dependent effect. An effective dose range is between 5 and 500 mg/kg of body weight/per day.

Preliminary assessment of the plasma level of the precursor of inflammation-associated amyloidosis, SAA, has shown that there is no difference between the animals being treated with PVS and those untreated.

The method of administering the agents of the present invention is believed to have had an effect upon the mortality rate of the animals. Intraperitoneal injection was selected as providing a large membrane surface for ease of access to the circulating system. However, like heparan, the compounds of the present invention exhibit anti-coagulant properties. Repeated injections through the peritoneal wall induced severe hemorrhaging and ultimately resulted in filling the peritoneal cavity, with loss of blood causing death. While subcutaneous injection would result in slower absorption of the active compound, it is less likely that this route would cause hemorrhaging to such an extent as to cause death. Oral administration of the compounds was performed in subsequent experiments (see below).

Experiment 2

Swiss white mice weighing 25-30 g were given Amyloid Enhancing Factor (AEF) and $AgNO_3$ as described previously (Kisilevsky, R. and Boudreau, L. (1983) "The kinetics of amyloid deposition: I. The effect of amyloid enhancing factor and splenectomy" *Lab. Invest.*, 48, 53-59), to induce amyloidosis. Twenty four (24) hours later they were divided into three groups. One group served as a control and was maintained on standard laboratory mouse chow and tap water *ad lib*. A second group received the standard chow but

its water contained 20 mg/ml of poly(vinyl sulfonate) sodium salt (PVS). The third group had 50 mg/ml of PVS in its drinking water. Fluid intake in both groups was the same. All animals were sacrificed on day six (6) of the experiment, their spleens collected, prepared for sectioning, spleen sections stained with Congo red (Puchtler, H., *et al.* (1983) "Application of Thiazole Dyes to Amyloid under Conditions of Direct Cotton Dyeing: Correlation of Histochemical and Chemical Data" *Histochemistry*, 77, 431-445), and the percent area occupied by amyloid assessed by an image analysis apparatus and program (MCID M2, Imaging Research Inc., Brock University, St. Catherines, Ontario, Canada). As shown in Figure 4, oral administration of PVS interferes with amyloid deposition in a dose dependent manner.

Experiment 3

Since it was possible that PVS was inhibiting the hepatic synthesis of the amyloid precursor, and thus failure to deposit amyloid was due to the absence of the precursor pool, the effect of PVS on the blood level of the amyloid precursor (SAA) during the course of the experiment was determined. Animals received AEF + $AgNO_3$ as described above and were divided into two groups. Group 1 received no further treatment. Twenty four hours later, Group 2 received 50 mg of PVS by intraperitoneal injection every 12 hours for a period of 5 days. To plot the level of SAA during this process, each animal (controls and experimentals) was bled from the tail (≈25 µl) each day. The SAA levels in these samples were determined by a solid phase ELISA procedure (described in Brissette, L., et al. (1989) *J. Biol. Chem.*, 264, 19327-19332). The results are shown in Figure 5. The open circles represent the data from the PVS-treated mice, while the triangles show the data from the non-treated animals. SAA levels were equivalent in treated and untreated animals, demonstrating that PVS does not mediate its effect by preventing the synthesis of SAA.

Experiment 4

In the above described experiments, therapy with PVS was begun 24 hours into the amyloid induction protocol. This does not mimic a clinical situation where the patient usually has well established amyloid. To approximate a more realistic clinical situation, a separate set of experiments were performed in which PVS treatment was begun after amyloid deposition had already begun. Animals received AEF + $AgNO_3$, as described above, remained on tap water for 7 days, after which they were separated into two groups. Group 1 remained on standard food and tap water. Group 2 remained on standard food but had 50 mg/ml of PVS added to their drinking water. To assess the effect of PVS on the course of amyloid deposition after amyloid was already present, five animals in each group were sacrificed on days 7, 10, 14, and 17. The spleens were processed and evaluated as described above. The data are shown in Figure 6. Control animals (triangles) continued to

deposit amyloid for 14 days, following which the quantity of amyloid began to decrease. This latter decrease is most likely due to the fact that only one injection of $AgNO_3$, the inflammatory stimulus, was given and, after 14 days, the SAA levels are known to decrease (Kisilevsky, R., Boudreau, L. and Foster, D. (1983) "Kinetics of amyloid deposition. II. The effects of dimethylsulfoxide and colchicine therapy" *Lab. Invest.*, 48, 60-67). In the absence of precursor, further amyloid cannot be deposited and existing deposits are mobilized (Kisilevsky, R. and Boudreau, L. (1983) "The kinetics of amyloid deposition: I. The effect of amyloid enhancing factor and splenectomy" *Lab. Invest.*, 48, 53-59). In contrast, the treated group of animals (open circles) stopped deposition of amyloid within 3 days of being placed on PVS. This demonstrates that PVS is effective at inhibiting ongoing deposition of amyloid.

Experiment 5

To maintain the inflammation and the blood SAA levels, and allow amyloid to be continuously deposited for the duration of a longer term experiment, the nature of the inflammatory stimulus was changed. So as to maintain the inflammation, animals received lipopolysaccharide (LPS, 20 μg)+ AEF on day 0 and LPS was given by intraperitoneal injection every 2nd day. On day seven (7), the animals were separated into two groups as described in Experiment 4. Assessment of amyloid over the course of the experiment proceeded as described in Experiment 4. The data are shown in Figure 7. The control group (triangles) continued to deposit amyloid for the entire 17 day period. Those receiving PVS apparently stopped depositing amyloid by day 14 (open circles and dashed line). The data on day 17 represent 4 animals per group as one animal was omitted from this time period. The quantity of amyloid in this particular individual was so far removed from all other data points (treated or not, it was 21%) that it is believed that this was a statistically valid procedure. If this individual is included, the curve is represented by the dotted line and the remaining open circle. It should be pointed out that animals receiving PVS began to develop a significant diarrhea as the experiment proceeded.

Experiment 6

In this experiment, another sulfonated compound, ethane monosulfate was used to inhibit amyloidosis. Ethane monosulfonate, sodium salt, (EMS) is structurally the monomeric unit of PVS. Animals were given LPS + AEF as in Experiment 5, but on day seven EMS was used in the drinking water as the therapeutic agent. On day seven, the animals were divided into three groups. Group 1 was the untreated group. Group 2 received 2.5 mg/ml EMS in their drinking water. Group 3 received 6 mg/ml in their drinking water. Animals were sacrificed on days 7, 10, 14, and 17. These animals did not develop gastro-intestinal problems. These data are shown in Figure 8. Animals receiving 6 mg/ml EMS in

their drinking water (open squares) stopped depositing amyloid after day 14. Those receiving 2.5 mg/ml EMS (open circles) seemed to have an abortive therapeutic effect, with a slight diminution in the rate of amyloid deposition at day 14 which was not maintained by day 17.

Experiment 7

The Influence of PVS on HSPG Binding to the Alzheimer's Amyloid Precursor Protein (Beta APP)

The binding of heparan sulfate proteoglycan to beta APP was assessed using an enzyme-linked immunosorbent assay technique as described in Narindrasorasak, S. et al. ("High Affinity Interactions Between the Alzheimer's Beta-Amyloid Precursor Proteins and the Basement Membrane Form of Heparan Sulfate Proteoglycan" *J. Biol. Chem.* 266:12878-12883 (1991)). Polystyrene microtiter plates (Linbro, Flow Laboratories) were coated with a 100 µl solution, 1 µg/ml of β-APP, in 20 mM $NaHCO_3$ buffer, pH 9.6. After overnight incubation at 4 °C, the plates were rinsed with 0.15 M NaCl, 20 mM Tris-Cl, pH 7.5 (TBS). The plates were then incubated with 150 µl of 1 % bovine serum albumin (BSA) in TBS for 2 hours at 37 °C to block the residual hydrophobic surface on the wells. After rinsing with TBS containing 0.05 % (w/v) Tween 20 (TBS-Tween), 100 µl of various concentrations of HSPG in TBS-Tween were added alone or 500 µg/ml of PVS, either in Tris-buffered saline (TBS) or phosphate-buffered saline (PBS), was included in the binding assay to assess the effect of PVS on HSPG binding to β-APP. The plates were left overnight at 4 °C to permit maximum binding of HSPG to β-APP. The plates were then washed extensively and incubated 2 hours at 37 °C with 100 µl of anti-HSPG diluted in TBS-Tween containing 0.1 % BSA. The plates were washed again and incubated for another 2 hours with 100 µl of goat anti-rabbit IgG conjugated with alkaline phosphatase (1:2000 dilution) in TBS-Tween containing BSA as above. Finally, after further washing, the bound antibodies were detected by adding an alkaline phosphatase substrate solution (100 µl) containing 2 mg/ml *p*-nitrophenyl phosphate, 0.1 mM $ZnCl_2$, 1 mM $MgCl_2$, and 100 mM glycine, pH 10. The plates were left at room temperature for 15-120 minutes. The enzyme reaction was stopped by addition of 50 µl of 2 M NaOH. The absorbence of the released *p*-nitrophenyl was measured at 405 nm with a Titertek Multiscan/MCC 340 (Flow Laboratories). The amounts of HSPG bound were determined by the net A_{405} after subtracting the A from blank wells in which the HSPG incubation step was omitted. The effect of PVS on HSPG: beta-APP binding is illustrated in Figure 2 (in TBS) and Figure 3 (in PBS). Approximately 30-50% inhibition of binding is demonstrated with this compound.

Experiment 8

Acute amyloidosis was elicited in mice with $AgNO_3$ and amyloid enhancing factor as described in Experiment 2. Twenty-four hours later, the animals were divided into a control group and six test groups. The control group was maintained on standard laboratory mouse chow and tap water *ad. lib.* The test groups received standard chow but their water contained 50 μM of one of the following six compounds: ethanesulfonic acid sodium salt, 2-amino-ethanesulfonic acid sodium salt (taurine), propane-1-sulfonic acid sodium salt, ethane-1,2-disulfonic acid sodium salt, propane-1,3-disulfonic acid sodium salt, or butane-1,4-disulfonic acid sodium salt. Water intake was approximately equivalent for all groups. After six days, the animals were sacrificed and their spleens were processed as described for Experiment 2. For preliminary analysis, the spleen sections were examined visually under a microscope for differences in amyloid deposition in the treated animals versus the control animals.

The results indicated that animals treated with propane-1-sulfonic acid sodium salt, ethane-1,2-disulfonic acid sodium salt, or propane-1,3-disulfonic acid sodium salt had less amyloid deposition than control animals. Under these acute amyloidosis conditions, animals treated with ethanesulfonic acid sodium salt, taurine sodium salt, or butane-1,4-disulfonic acid sodium salt were not observed to have less amyloid deposition than control animals. However, these compounds may exhibit effectiveness under other conditions, for example ethanesulfonic acid sodium salt has been observed to inhibit chronic amyloid deposition (see Experiment 6).

This experiment suggests that oral administration of sulfonated lower aliphatics such as propane-1-sulfonic acid sodium salt, ethane-1,2-disulfonic acid sodium salt and propane-1,3-disulfonic acid sodium salt can inhibit amyloid deposition in an acute amyloidogenic system.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.

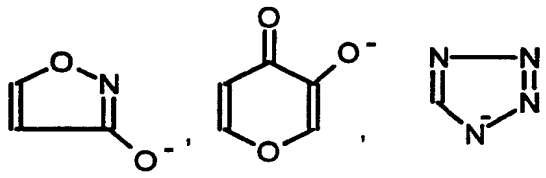
CLAIMS

1. A method for inhibiting amyloid deposition in a subject comprising administering to the subject an effective amount of a therapeutic compound, the therapeutic compound comprising at least one anionic group attached to a carrier molecule, or a pharmaceutically acceptable salt thereof, such that the therapeutic compound inhibits an interaction between an amyloidogenic protein and a glycoprotein or proteoglycan constituent of a basement membrane to inhibit amyloid deposition.

2. The method of claim 1 further comprising administering the therapeutic compound in a pharmaceutically acceptable vehicle.

3. The method of claim 1, wherein the carrier molecule is selected from the group consisting of a carbohydrate, a polymer, a peptide, a peptide derivative, an aliphatic group, an alicyclic group, an heterocyclic group, an aromatic group and combinations thereof.

4. The method of claim 1, wherein the anionic group is selected from the group consisting of a sulfonate group, a sulfate group, a carboxylate group, a phosphate group, a phosphonate group, and a heterocyclic group selected from the group consisting of



5. A method for inhibiting amyloid deposition in a subject comprising administering to the subject an effective amount of a therapeutic compound, the therapeutic compound comprising at least one sulfonate group covalently attached to a carrier molecule, or a pharmaceutically acceptable salt thereof, such that the therapeutic compound inhibits an interaction between an amyloidogenic protein and a glycoprotein or proteoglycan constituent of a basement membrane to inhibit amyloid deposition.

6. A method for inhibiting amyloid deposition in a subject comprising administering to the subject an effective amount of a therapeutic compound, the therapeutic compound having the following formula:

$$Q\text{—}[\text{—}SO_3^-X^+]_n$$

wherein Q is a carrier molecule; X^+ is a cationic group; and n is an integer selected such that the biodistribution of the therapeutic compound for an intended target site is not prevented while maintaining activity of the therapeutic compound, wherein the therapeutic compound inhibits an interaction between an amyloidogenic protein and a glycoprotein or proteoglycan constituent of a basement membrane to inhibit amyloid deposition.

7. The method of claim 5, wherein the therapeutic compound is administered orally.

8. The method of claim 5, further comprising administering the therapeutic compound in a pharmaceutically acceptable vehicle.

9. The method of claim 5, wherein the carrier molecule is selected from the group consisting of a carbohydrate, a polymer, a peptide, a peptide derivative, an aliphatic group, an alicyclic group, a heterocyclic group, an aromatic group and combinations thereof.

10. The method of claim 5, wherein the carrier molecule is a carbohydrate.

11. The method of claim 5, wherein the aliphatic group is a lower aliphatic.

12. The method of claim 5, wherein the carrier molecule is a polymer.

13. The method of claim 12, wherein the polymer is selected from the group consisting of substituted and unsubstituted vinyl, acryl, styrene and carbohydrate-derived polymers and copolymers and pharmaceutically acceptable salts thereof.

14. A method for inhibiting amyloid deposition in a subject comprising orally administering to the subject an effective amount of a therapeutic compound, the therapeutic compound comprising at least one sulfonate group covalently attached to a carrier molecule, or a pharmaceutically acceptable salt thereof.

15. The method of claim 14 further comprising administering the therapeutic compound in a pharmaceutically acceptable vehicle.

16. The method of claim 14, wherein the carrier molecule is selected from the group consisting of a carbohydrate, a polymer, a peptide, a peptide derivative, an aliphatic group, an alicyclic group, a heterocyclic group, an aromatic group and combinations thereof.

17. A method for inhibiting amyloid deposition in a subject comprising administering to the subject an effective amount of a therapeutic compound, the therapeutic compound comprising at least one sulfate group covalently attached to a carrier molecule, or a pharmaceutically acceptable salt thereof, such that the therapeutic compound inhibits an interaction between an amyloidogenic protein and a glycoprotein or proteoglycan constituent of a basement membrane to inhibit amyloid deposition.

18. A method for inhibiting amyloid deposition in a subject comprising administering to the subject an effective amount of a therapeutic compound, the therapeutic compound having the following formula:

$$Q—[—OSO_3^-X^+]_n$$

wherein Q is a carrier molecule; X^+ is a cationic group; and n is an integer selected such that the biodistribution of the therapeutic compound for an intended target site is not prevented while maintaining activity of the therapeutic compound, wherein the therapeutic compound inhibits an interaction between an amyloidogenic protein and a glycoprotein or proteoglycan constituent of a basement membrane to inhibit amyloid deposition.

19. The method of claim 17, wherein the therapeutic compound is administered orally.

20. The method of claim 17, further comprising administering the therapeutic compound in a pharmaceutically acceptable vehicle.

21. The method of claim 17, wherein the carrier molecule is selected from the group consisting of a carbohydrate, a polymer, a peptide, a peptide derivative, an aliphatic group, an alicyclic group, a heterocyclic group, an aromatic group and combinations thereof.

22. The method of claim 17, wherein the carrier molecule is a carbohydrate.

23. The method of claim 17, wherein the aliphatic group is a lower aliphatic.

24. The method of claim 17, wherein the carrier molecule is a polymer.

25. The method of claim 24, wherein the polymer is selected from the group consisting of substituted and unsubstituted vinyl, acryl, styrene and carbohydrate-derived polymers and copolymers and pharmaceutically acceptable salts thereof.

26. A method of inhibiting amyloid deposition in a subject comprising administering to the subject an effective amount of a therapeutic compound comprising at least one sulfonate groups covalently attached to a lower aliphatic group, or a pharmaceutically acceptable salt thereof, optionally in a pharmaceutically acceptable vehicle.

27. The method of claim 26, wherein the therapeutic compound is selected from the group consisting of ethanesulfonic acid, ethane-1,2-disulfonic acid, propane-1-sulfonic acid, propane-1,2-disulfonic acid and pharmaceutically acceptable salts thereof.

28. The method of claim 26, wherein the therapeutic compound is administered orally.

29. A method of inhibiting amyloid deposition in a subject comprising administering to the subject an effective amount of a therapeutic compound comprising at least one sulfonate groups covalently attached to a disaccharide, or a pharmaceutically acceptable salt thereof, optionally in a pharmaceutically acceptable vehicle.

30. The method of claim 29, wherein the disaccharide is sucrose.

31. A method of inhibiting amyloid deposition in a subject comprising administering to the subject an effective amount of a therapeutic compound comprising at least one sulfonate groups covalently attached to a polymer, or a pharmaceutically acceptable salt thereof, optionally in a pharmaceutically acceptable vehicle.

32. The method of claim 31, wherein the therapeutic compound is selected from the group consisting of poly(2-acrylamido-2-methyl-1-propanesulfonic acid); poly(2-acrylamido-2-methyl-1-propanesulfonic acid-co-acrylonitrile); poly(2-acrylamido-2-methyl-1-propanesulfonic acid-co-styrene); poly(vinylsulfonic acid); poly(sodium 4-styrenesulfonic acid); a sulfonate derivative of poly(acrylic acid); a sulfonate derivative of poly(methyl acrylate); a sulfonate derivative of poly(methyl methacrylate); and pharmaceutically acceptable salts thereof.

33. The method of claim 31, wherein the therapeutic compound is poly(vinyl sulfonic acid) or a pharmaceutically acceptable salt thereof.

34. A method of inhibiting amyloid deposition in a subject comprising administering to the subject an effective amount of a therapeutic compound comprising at

least one sulfonate groups covalently attached to a peptide and a peptide derivative, or a pharmaceutically acceptable salt thereof, optionally in a pharmaceutically acceptable vehicle.

35. A method of inhibiting amyloid deposition in a subject comprising administering to the subject an effective amount of a therapeutic compound comprising at least one sulfate groups covalently attached to a lower aliphatic group, or a pharmaceutically acceptable salt thereof, optionally in a pharmaceutically acceptable vehicle.

36. A method of inhibiting amyloid deposition in a subject comprising administering to the subject an effective amount of a therapeutic compound comprising at least one sulfate groups covalently attached to a disaccharide, or a pharmaceutically acceptable salt thereof, optionally in a pharmaceutically acceptable vehicle.

37. The method of claim 36, wherein the therapeutic compound is sucrose octasulfate or a pharmaceutically acceptable salt thereof.

38. A method of inhibiting amyloid deposition in a subject comprising administering to the subject an effective amount of a therapeutic compound comprising at least one sulfate groups covalently attached to a polymer, or a pharmaceutically acceptable salt thereof, optionally in a pharmaceutically acceptable vehicle.

39. The method of claim 38, wherein the therapeutic compound is selected from the group consisting of poly(2-acrylamido-2-methyl-1-propanesulfuric acid); poly(2-acrylamido-2-methyl-1-propanesulfuric acid-co-acrylonitrile); poly(2-acrylamido-2-methyl-1-propanesulfuric acid-co-styrene); poly(vinylsulfuric acid); poly(sodium 4-styrenesulfuric acid); a sulfate derivative of poly(acrylic acid); a sulfate derivative of poly(methyl acrylate); a sulfate derivative of poly(methyl methacrylate); and pharmaceutically acceptable salts thereof.

40. A method of inhibiting amyloid deposition in a subject comprising administering to the subject an effective amount of a therapeutic compound comprising at least one sulfate groups covalently attached to a peptide and a peptide derivative, or a pharmaceutically acceptable salt thereof, optionally in a pharmaceutically acceptable vehicle.

41. A pharmaceutical composition for treating amyloidosis comprising a therapeutic compound comprising at least one sulfonate group covalently attached to a carrier molecule, or a pharmaceutically acceptable salt thereof, in an amount sufficient to inhibit an

interaction between an amyloidogenic protein and a basement membrane constituent; and a pharmaceutically acceptable vehicle.

42. The pharmaceutical composition of claim 41, wherein the carrier molecule is selected from the group consisting of a carbohydrate, a polymer, a peptide, a peptide derivative, an aliphatic group, an alicyclic group, a heterocyclic group, an aromatic group and combinations thereof.

43. A pharmaceutical composition for treating amyloidosis comprising a therapeutic compound comprising at least one sulfate group covalently attached to a carrier molecule, or a pharmaceutically acceptable salt thereof, in an amount sufficient to inhibit an interaction between an amyloidogenic protein and a basement membrane constituent, and a pharmaceutically acceptable vehicle.

44. The pharmaceutical composition of claim 43, wherein the carrier molecule is selected from the group consisting of a carbohydrate, a polymer, a peptide, a peptide derivative, an aliphatic group, an alicyclic group, a heterocyclic group, an aromatic group and combinations thereof.

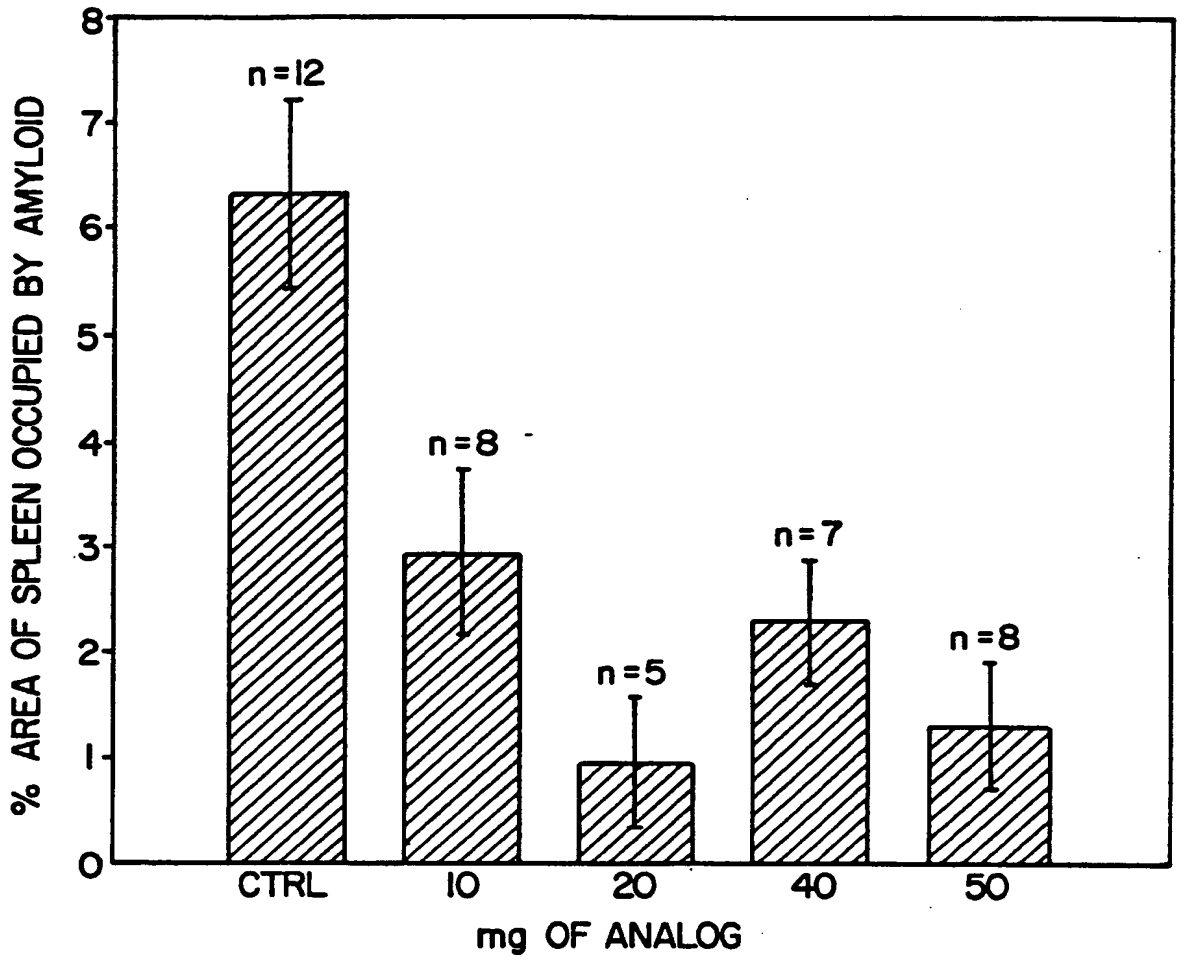
45. A pharmaceutical composition for treating amyloidosis comprising poly(vinyl sulfonic acid), or a pharmaceutically acceptable salt thereof, in an amount sufficient to inhibit an interaction between an amyloidogenic protein and a basement membrane constituent, and a pharmaceutically acceptable vehicle.

46. A pharmaceutical composition for treating amyloidosis comprising ethanesulfonic acid, or a pharmaceutically acceptable salt thereof, in an amount sufficient to inhibit , an interaction between an amyloidogenic protein and a basement membrane constituent, and a pharmaceutically acceptable vehicle.

47. A pharmaceutical composition for treating amyloidosis comprising sucrose octasulfate, or a pharmaceutically acceptable salt thereof, in an amount sufficient to inhibit , an interaction between an amyloidogenic protein and a basement membrane constituent, and a pharmaceutically acceptable vehicle.

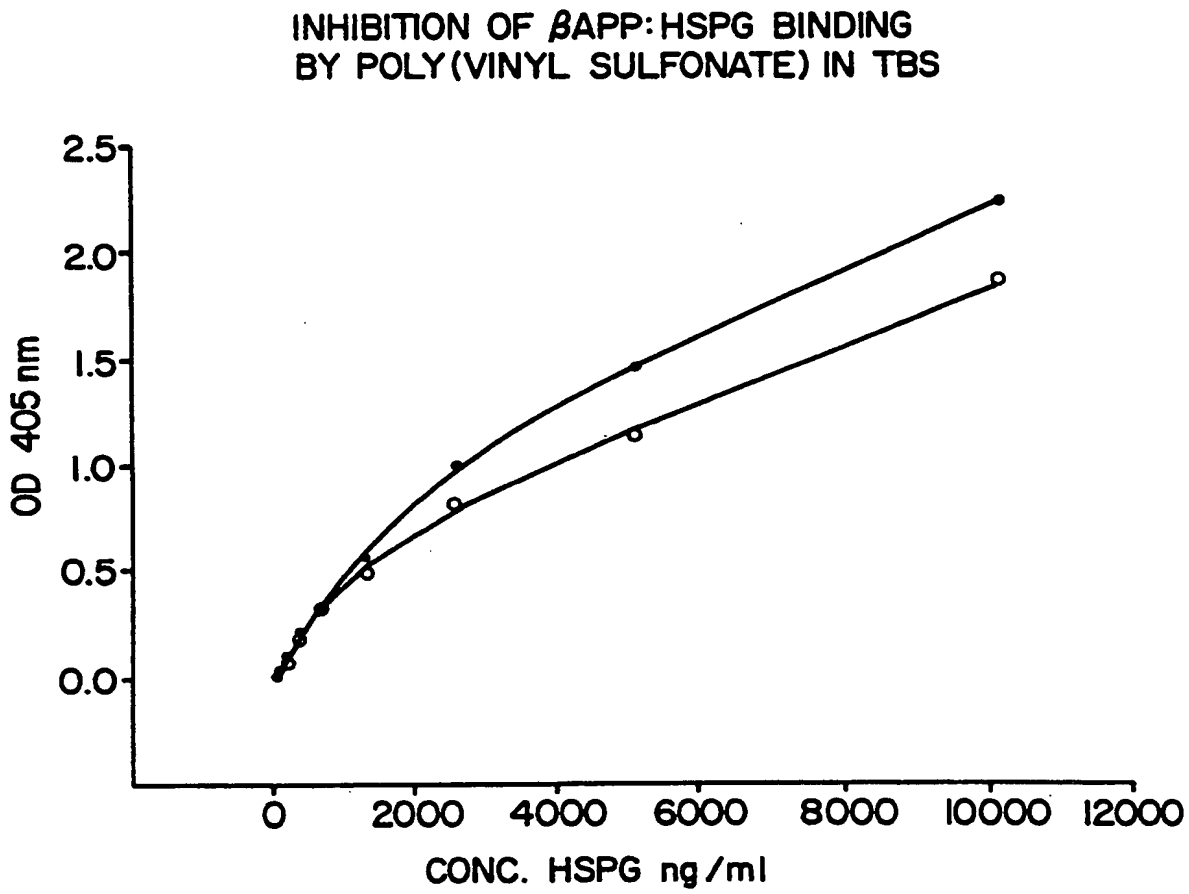
1/6

EFFECT OF POLY(VINYL SULFONATE)
ON IN VIVO AA SPLENIC AMYLOID DEPOSITION



VALUES REPRESENT THE MEAN ± S.E.M. OF n DETERMINATIONS

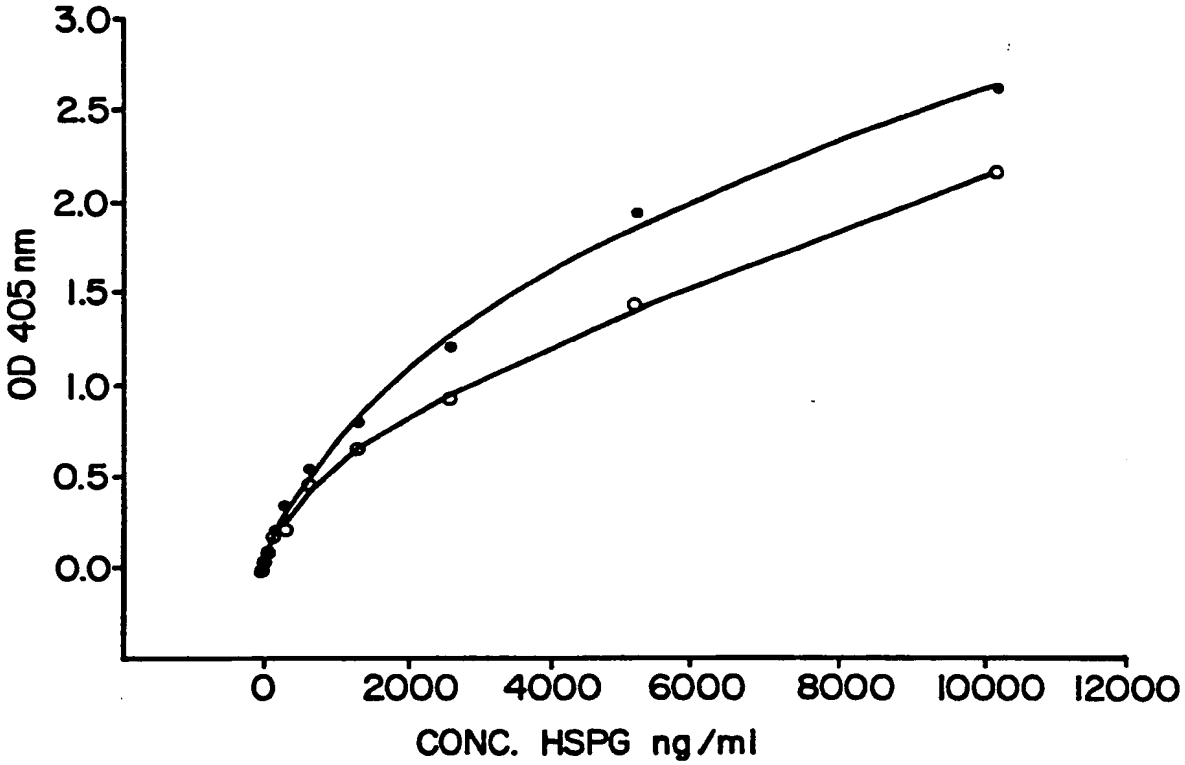
FIG. 1



	K_d	B_{max}
• HSPG	6nM	1.42
○ HSPG + POLY(VINYL SULFONATE)	2nM	0.675

FIG. 2

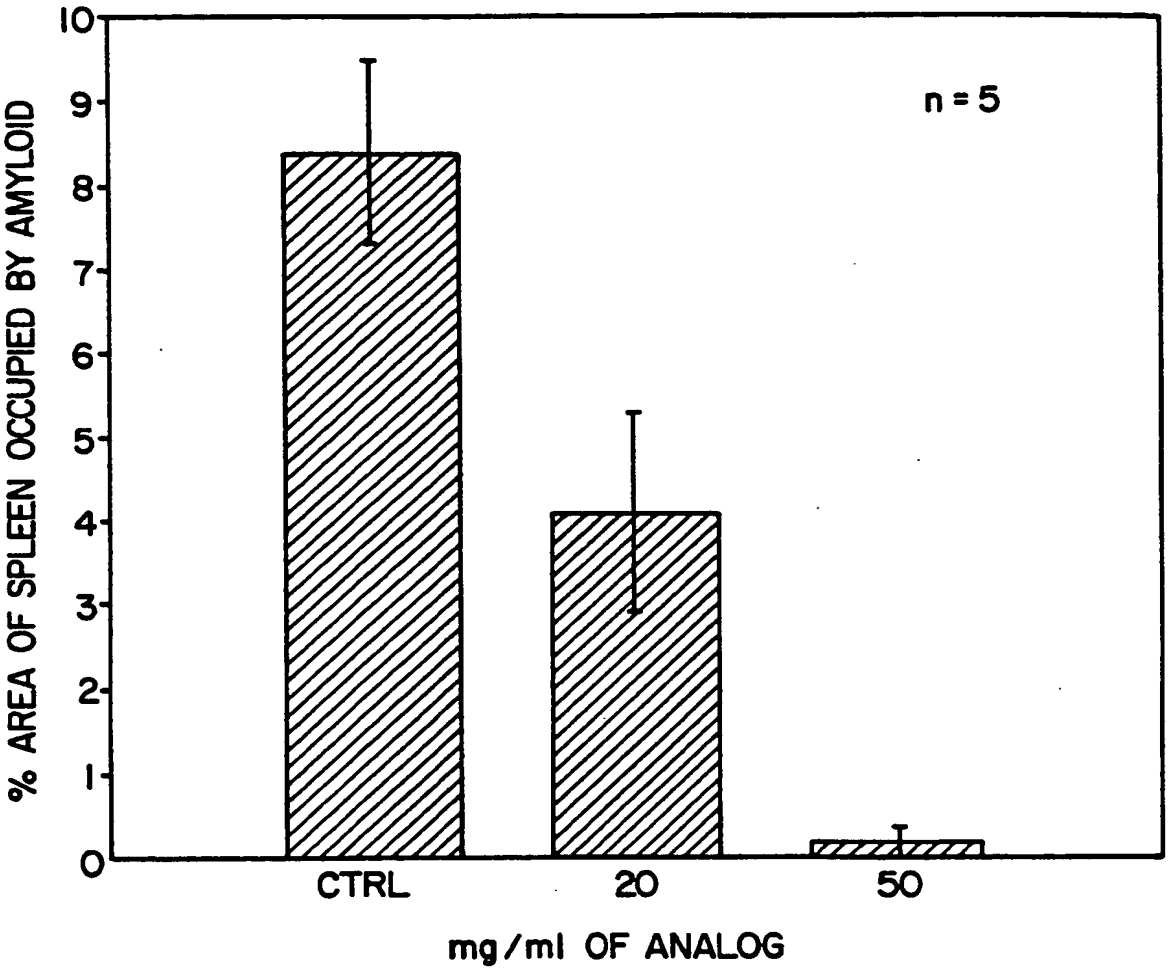
INHIBITION OF βAPP: HSPG BINDING
BY POLY(VINYL SULFONATE) IN PBS



	K_d	B_{max}
• HSPG	5nM	1.94
○ HSPG + POLY(VINYL SULFONATE)	2nM	0.859

FIG. 3

EFFECT OF POLY(VINYL SULFONATE)
ON IN VIVO AA SPLENIC AMYLOID DEPOSITION



VALUES REPRESENT THE MEAN ± S.E.M. OF n DETERMINATIONS

FIG. 4

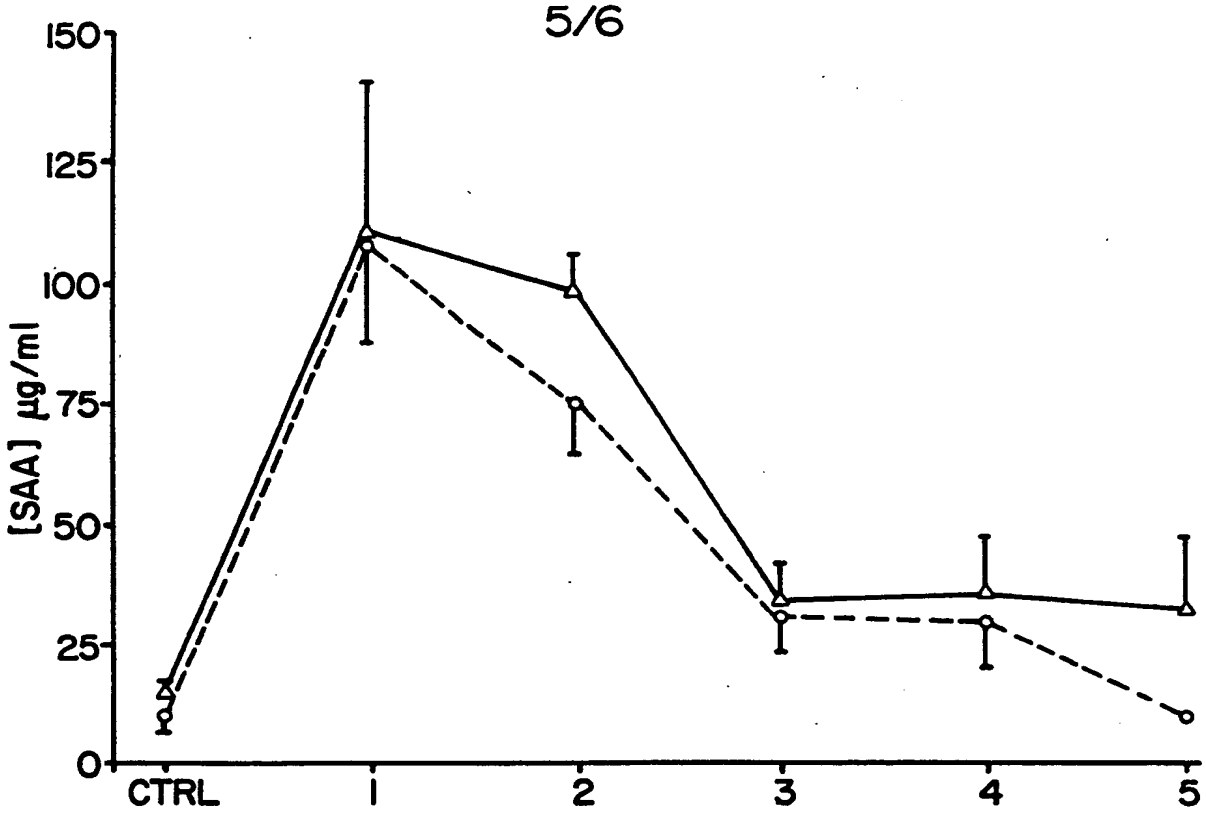


FIG. 5

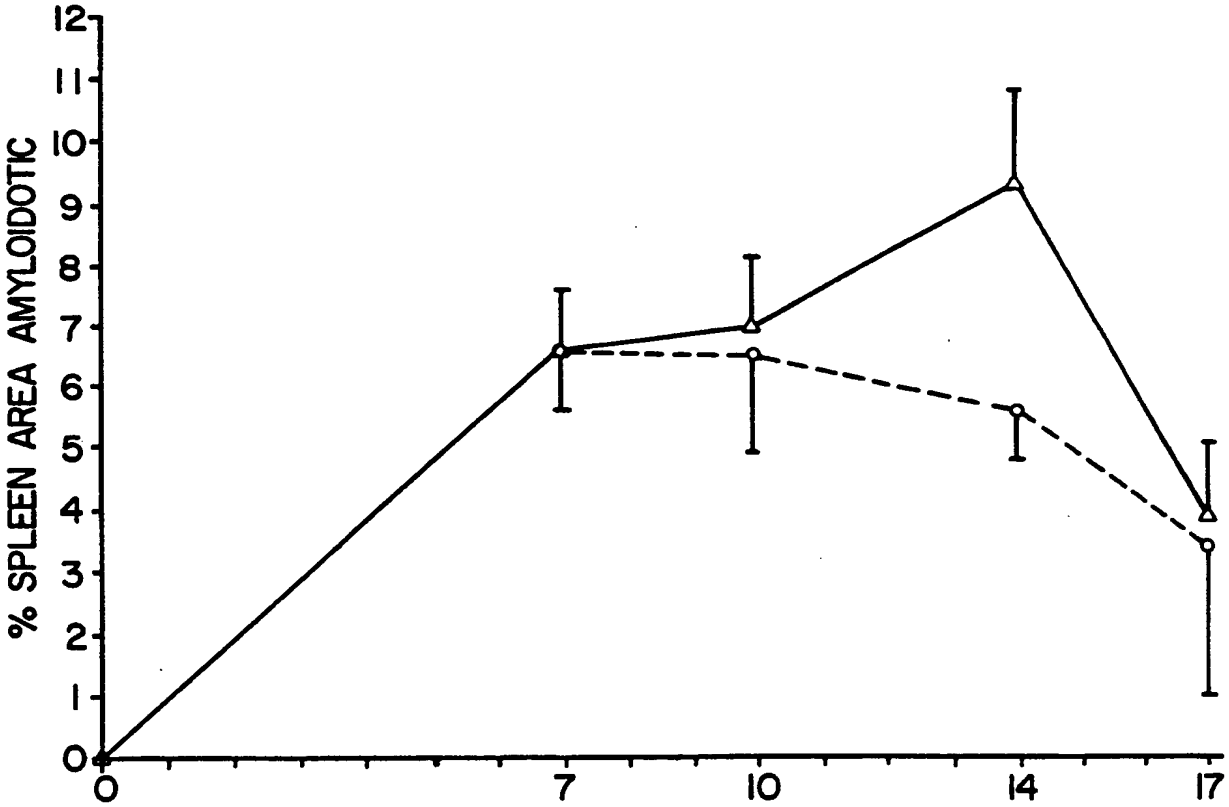


FIG. 6

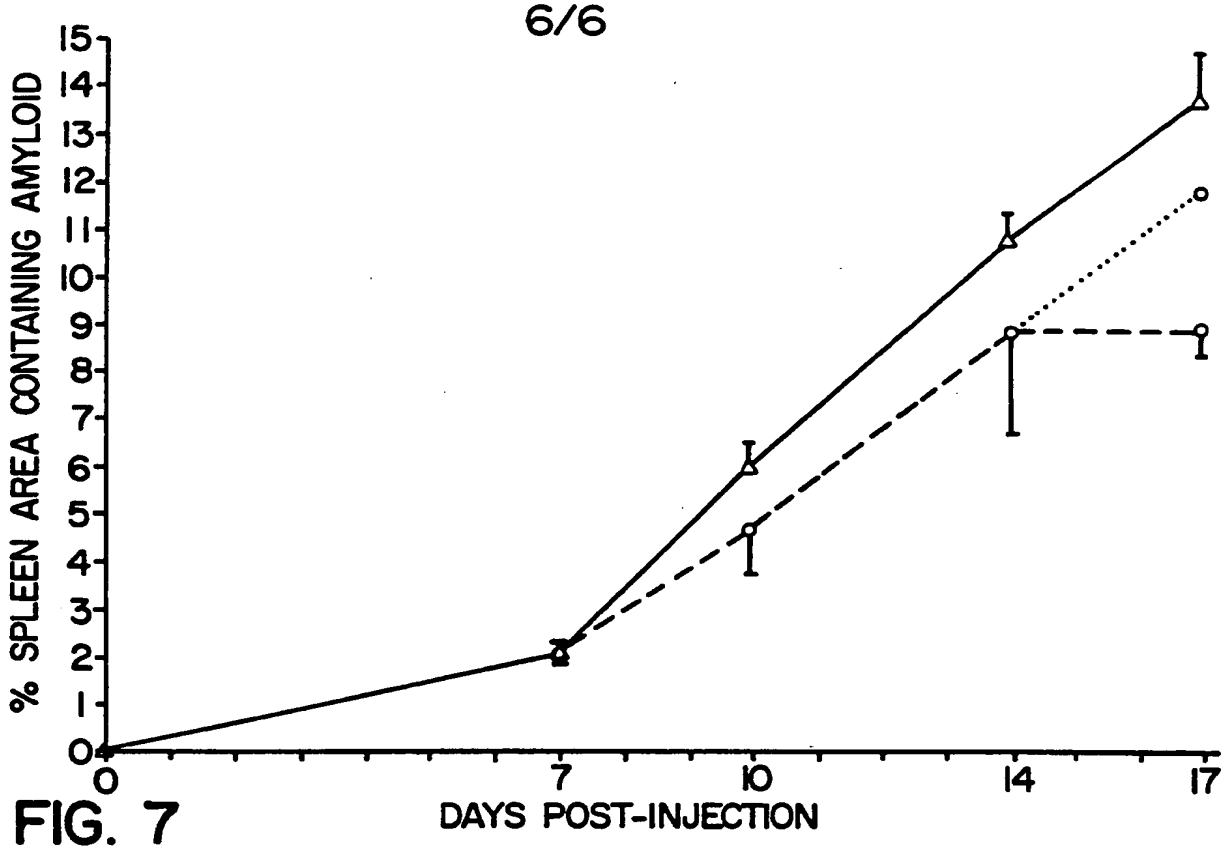


FIG. 7

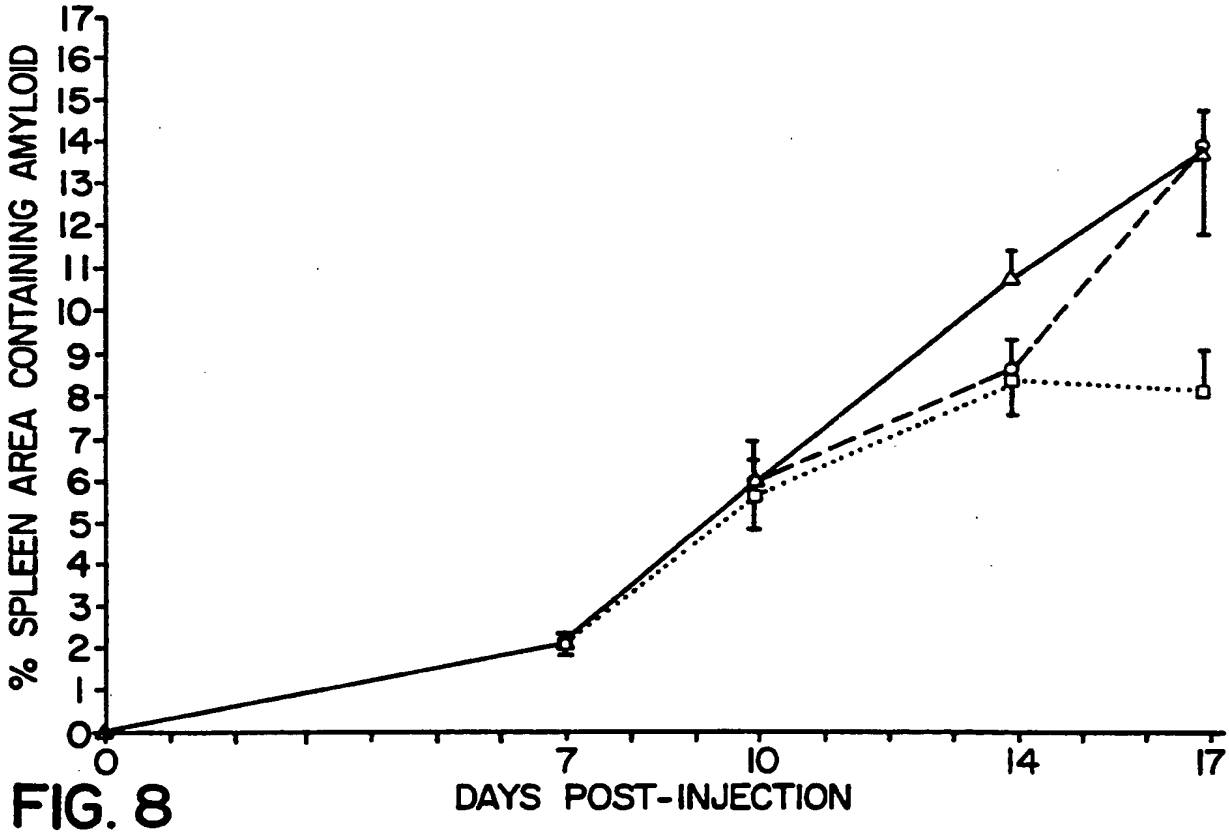


FIG. 8

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(71) Applicant: QUEEN'S UNIVERSITY AT KINGSTON [CA/CA]; Kingston, Ontario K7L 3N6 (CA).

(72) Inventors: KISILEVSKY, Robert; 120 Cairn Grove, Kingston, Ontario K7M 4B9 (CA). SZAREK, Walter, A.; 403-322 Brock Street, Kingston, Ontario K7L 1S9 (CA). WEAVER, Donald; 27 Collegeview Crescent, Kingston, Ontario K7M 7J8 (CA).

(74) Agent: HICKS, Richard, J.; Parteq Innovations, Queen's University, Kingston, Ontario K7L 3N6 (CA).

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(54) Title: METHOD FOR TREATING AMYLOIDOSIS

(57) Abstract

Therapeutic compounds and methods for inhibiting amyloid deposition in a subject, whatever its clinical setting, are described. Amyloid deposition is inhibited by the administration to a subject of an effective amount of a therapeutic compound comprising an anionic group and a carrier molecule, or a pharmaceutically acceptable salt thereof, such that an interaction between an amyloidogenic protein and a basement membrane constituent is inhibited. Preferred anionic groups are sulfonates and sulfates. Preferred carrier molecules include carbohydrates, polymers, peptides, peptide derivatives, aliphatic groups, alicyclic groups, heterocyclic groups, aromatic groups and combinations thereof.

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A. CLASSIFICATION OF SUBJEC TTER
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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	NEUROREPORT, vol.4, no.8, August 1993 pages 1035 - 1038 T. NAKADA ET AL. 'GUANIDINOETHANE SULFATE: BRAIN pH ALKALINE SHIFTER' see page 1038 ---	1-9,11, 14,26,28
X	US,A,5 164 295 (KISILEVSKY ET AL.) 17 November 1992 cited in the application see the whole document --- -/--	1-10, 12-22, 24,25, 31,38, 41-44

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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2

Date of the actual completion of the international search	Date of mailing of the international search report
1 September 1994	27. 12. 94
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer HOFF, P

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol.266, no.20, 1991 pages 12878 - 12883 S. NARINDRASORASAK ET AL. 'HIGH AFFINITY INTERACTIONS BETWEEN THE ALZHEIMER S beta-AMYLOID PRECURSOR PROTEINS AND THE BASEMENT MENBRANE FORM OF HEPARAN SULFATE PROTEOGLYCAN' see the whole document ---	1-10, 12-22, 24,25, 31,38, 41-44
X	JOURNAL OF VIROLOGY, vol.67, no.2, February 1993 pages 643 - 650 B. CAUGHEY ET AL. 'SULFATED POLYANION INHIBITION OF SCRAPIE-ASSOCIATED PrP ACCUMULATION IN CULTURED CELLS'	1-10, 12-22, 24,25, 31,38, 41-44
Y	see the whole document ---	32,33, 39,45
Y	PATENT ABSTRACTS OF JAPAN vol. 01, no. 3447 (C-642) 6 October 1989 & JP,A,01 171 638 (KANEGAFUCHI) 6 July 1989 see abstract ---	32,33, 39,45
X	BIOCHIMICA ET BIOPHYSICA ACTA, vol.998, no.3, 1989 pages 231 - 235 H. HAMAZKI 'CALCIUM-DEPENDENT POLYMERIZATION OF HUMAN SERUM AMYLOID P COMPONENT IS INHIBITED BY HEPARIN AND DEXTRAN SULFATE' see the whole document ---	1-10, 12-22, 24,25, 31,38, 41-44
X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol.262, no.4, 1987 pages 1456 - 1460 H. HAMAZAKI 'CA2+-MEDIATED ASSOCIATION OF HUMAN SERUM AMYLOID P COMPONENT WITH HEPARAN SULFATE AND DERMATAN SULFATE' see the whole document ---	1-10, 12-22, 24,25, 31,38, 41-44
P,X	WO,A,94 01116 (THE GOVERNMENT OF THE UNITED STATES) 20 January 1994 see the whole document ---	1-9, 14-16, 41,42
X	WO,A,90 09789 (AVERBACK) 7 September 1990 see abstract; claims; example 3 ---	1-9, 14-16, 41,42
11 X	'THE MERCK INDEX' , MERCK & CO., INC. , RAHWAY, N.J., USA see page 883 ---	41,42,45

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,89 05646 (BAR-SHALOM) 29 June 1989 see abstract; claims 1,8,19,20,27	43,44,47
A	EP,A,0 293 974 (CRINOS INDUSTRIA FARMACOBIOLOGICA) 7 December 1988 cited in the application see the whole document	1-47

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA94/00160

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Claims: 1-4 (partially), and 5-47 (completely).
Claims: 1-4 (partially)
Claims: 1-4 (partially)
Claims: 1-4 (partially)

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invenuon first mentioned in the claims; it is covered by claims Nos.:

1-4 (partially), and 5-47 (completely).

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
US-A-5164295	17-11-92	NONE		
WO-A-9401116	20-01-94	US-A-	5276059	04-01-94
		AU-B-	4678193	31-01-94
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		CA-A-	2032141	28-08-90
		EP-A-	0460078	11-12-91
		JP-T-	4505006	03-09-92
		US-A-	5231170	27-07-93
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		JP-T-	4500798	13-02-92
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